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International Journal of Food Microbiology 83 (2003) 147–160
INTERNATIONAL JOURNAL OF Food Microbiology
www.elsevier.com/locate/ijfoodmicro

Influence of agitation, inoculum density, pH, and strain on the growth parameters of *Escherichia coli* O157:H7—relevance to risk assessment☆

M.E. Coleman[a,b,*], M.L. Tamplin[b], J.G. Phillips[c], B.S. Marmer[b]

[a] *USDA/Food Safety and Inspection Service, Office of Public Health and Science, Food Risk Assessment Center, 1400 Independence Ave., SW, Rm. 386 Aerospace Building, Washington, DC 20250-3700, USA*

[b] *Microbial Food Safety Research, United States Department of Agriculture, Agricultural Research Service, Eastern Regional Research Center, Wyndmoor, PA 19038-8598, USA*

[c] *North Atlantic Area, 600 East Mermaid Lane, Wyndmoor, PA 19038-8598, USA*

Received 21 March 2002; received in revised form 9 July 2002; accepted 16 July 2002

Abstract

Foods may differ in at least two key variables from broth culture systems typically used to measure growth kinetics of enteropathogens: initial population density of the pathogen and agitation of the culture. The present study used nine *Escherichia coli* O157:H7 strains isolated from beef and associated with human illness. Initial kinetic experiments with one *E. coli* O157:H7 strain in brain–heart infusion (BHI) broth at pH 5.5 were performed in a $2 \times 2 \times 3$ factorial design, testing the effects of a low (ca. 1–10 colony-forming units [CFU]/ml) or high (ca. 1000 CFU/ml) initial population density, culture agitation or no culture agitation, and incubation temperatures of 10, 19, and 37 °C. Kinetic data were modeled using simple linear regression and the Baranyi model. Both model forms provided good statistical fit to the data (adjusted r^2>0.95). Significant effects of agitation and initial population density were identified at 10 °C but not at 19 or 37 °C. Similar growth patterns were observed for two additional strains tested under the same experimental design. The lag, slope, and maximum population density (MPD) parameters were significantly different by treatment. Further tests were conducted in a 96-well microtiter plate system to determine the effect of initial population density and low pH (4.6–5.5) on the growth of *E. coli* O157:H7 strains in BHI at 10, 19, and 37 °C. Strain variability was more apparent at the boundary conditions of growth of low pH and low temperature. This study demonstrates the need for growth models that are specific to food products and environments for plausible extrapolation to risk assessment models.

Keywords: Predictive microbiology; Exposure assessment; Risk analysis

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* Corresponding author. USDA/Food Safety and Inspection Service, Office of Public Health and Science, Food Risk Assessment Center, 1400 Independence Ave., SW, Rm. 386 Aerospace Building, Washington, DC 20250-3700, USA. Tel.: +1-202-690-6468; fax: +1-202-690-6414.

E-mail address: Peg.Coleman@usda.gov (M.E. Coleman).

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doi:10.1016/S0168-1605(02)00367-7

1. Introduction

In conducting microbial risk assessments for many foods, extrapolation from culture broth models of microbial growth is required when kinetic data for specific food matrices are lacking. Key kinetic parameters for sigmoidal bacterial growth models include lag, a period of physiological adjustment of bacterial cells prior to growth, exponential growth rate, and maximum population density (MPD) reached in stationary growth phase, described ecologically as the carrying capacity of the population (Kot, 2001; Ross, 1999; McMeekin et al., 1993). Published microbial risk assessments for *Escherichia coli* O157:H7 in ground beef (Cassin et al., 1998; Marks, 1998; Powell et al., 2000) have utilized Gompertz model parameters based on microbial growth studies in broth culture (Buchanan and Klawitter, 1992; Buchanan et al., 1993) for exposure assessments of ground beef without adjustments for extrapolating from broth experiments to the food matrix. The typical experimental system for predictive microbiology growth studies is designed to measure growth under conditions including rich nutrient broth, relatively high initial densities of the target pathogen (e.g., 3–4 $\log_{10}$ CFU/ml), cocktails or mixtures of pure cultures of pathogenic strains, lack of competing microflora, and high levels of mixing, such as rotary shaking at 150 rpm. Another typical study system is the inoculated pack study, usually conducted at high initial densities of pathogens inoculated into a food matrix. Predictive microbiologists (Ross, 1999; Ross et al., 2000) expect that broth culture models are likely to be conservative, "fail-safe" systems that overpredict growth under more typical conditions of foods. Two major effects in broth culture protocols that might lead to overestimation bias were tested in this study, low initial densities typical of fresh ground beef (Marks, 1998) and agitation.

Depicting variability and uncertainty is of great importance for microbial risk assessment, but neither is well characterized for growth kinetics of bacteria in broth culture or food matrices (Nauta, 2002). This study protocol focuses on uncertainty in extrapolation of kinetic models for growth generated in culture broth to food matrices. The magnitude of the uncertainty associated with growth predictions extrapolated from culture broth models cannot be estimated without proper validation data or bridging studies, particularly for solid nonsterile foods such as ground beef associated with a dense indigenous spoilage microbiota (Ajjarapu and Shelef, 1999; USDA, 1996) that dominates the microbial ecology of this food at refrigeration temperatures. Particularly, as the boundary of the growth/no growth interface is approached near 10 °C for *E. coli* O157:H7 at low pH, uncertainty about how well the existing culture broth models (Buchanan and Klawitter, 1992; Buchanan et al., 1993) depict growth kinetics in foods may be high. Tamplin (in press) reported differences in growth parameters for this pathogen in ground beef at the lower temperature boundary that were less apparent in a previous study at a slightly higher temperature (Walls, 1996). However, neither study conducted experiments at the low initial densities estimated for this pathogen in fresh ground beef (Marks et al., 1998).

It is possible that exposure assessments for *E. coli* O157:H7 in ground beef based on kinetics of growth from fail-safe culture broth models without adjustment for food matrix effects may calculate biased predictions of growth for this pathogen in ground beef. Therefore, the current studies were designed to address four factors that may bias exposure assessment models for this pathogen: temperature, initial density of the pathogen, agitation or aeration, and strain.

2. Materials and methods

2.1. Cultures

Stock cultures of nine strains of *E. coli* O157:H7 were stored in brain–heart infusion (BHI; Becton, Dickinson Microbiology Systems, Sparks, MD) broth containing 10% glycerol at −70 °C. These strains were isolated from beef products associated with clinical illness and are designated as follows: OB1340, OB90520A, OB141412, OB1525C, OB1423C, OB1514C1, OB1680G, OB1533A, and DB1538; the strains were designated as strains 1 through 9, respectively.

2.2. Culture techniques

Stocks of BHI at various pH levels were prepared by adding concentrated HCl to levels 0.1 pH unit below the target pH. Following autoclaving, the final

pH was confirmed in an aliquot of the stock media. The target pH levels were 5.5 for the $2 \times 2 \times 3$ factorial experiment in 250-ml culture flasks. A further study was conducted as a 2×2 factorial experiment in the flask system at pH 5.5 or 4.6 and under shaken or unshaken conditions. For each experimental trial in the flask system, 49-ml BHI was dispensed into 8 to 16 sterile 250-ml flasks prior to inoculation and incubation. Microtiter plate experiments were conducted with BHI adjusted to target pH values from 4.6 to 5.4 in 0.2 pH unit increments and pH 5.5. For microtiter plate assays, 0.2 ml of BHI was dispensed using a multichannel pipetter (Rainin Instrument, Woburn, MA) into 96-well plates (Falcon U-bottom plates, BD Biosciences, San Jose, CA).

A loop of the frozen stock culture was transferred into 9 ml of fresh BHI (pH 7.4) and incubated at 37 °C without shaking for 4–6 h. This culture was then transferred with a loop into 9 ml of fresh BHI for 12–15 h of incubation at 37 °C without shaking. Cultures were adjusted aseptically with BHI (pH 5.5) to an optical density of 0.75–0.8 at 450 nm (approximately 8 $\log_{10}$ CFU/ml). Inocula were prepared from these working suspensions diluted in BHI (pH 5.5).

2.3. Experimental designs

The protocol for the initial study included a $2 \times 2 \times 3$ factorial design with duplicate flasks used for each combination of initial density (~ 3 $\log_{10}$ CFU/ml or 0–1 $\log_{10}$ CFU/ml) and agitation (shaken at 150 rpm or unshaken) at three incubation temperatures (10, 19, or 37 °C). Triplicate independent experiments were conducted at each temperature in separate weeks using strain 1. One flask per treatment was inoculated immediately with 1 ml of the working suspensions of diluted cultures. The working cultures were stored for 12 h at 4 °C prior to inoculation of the duplicate flask. This experimental design permitted

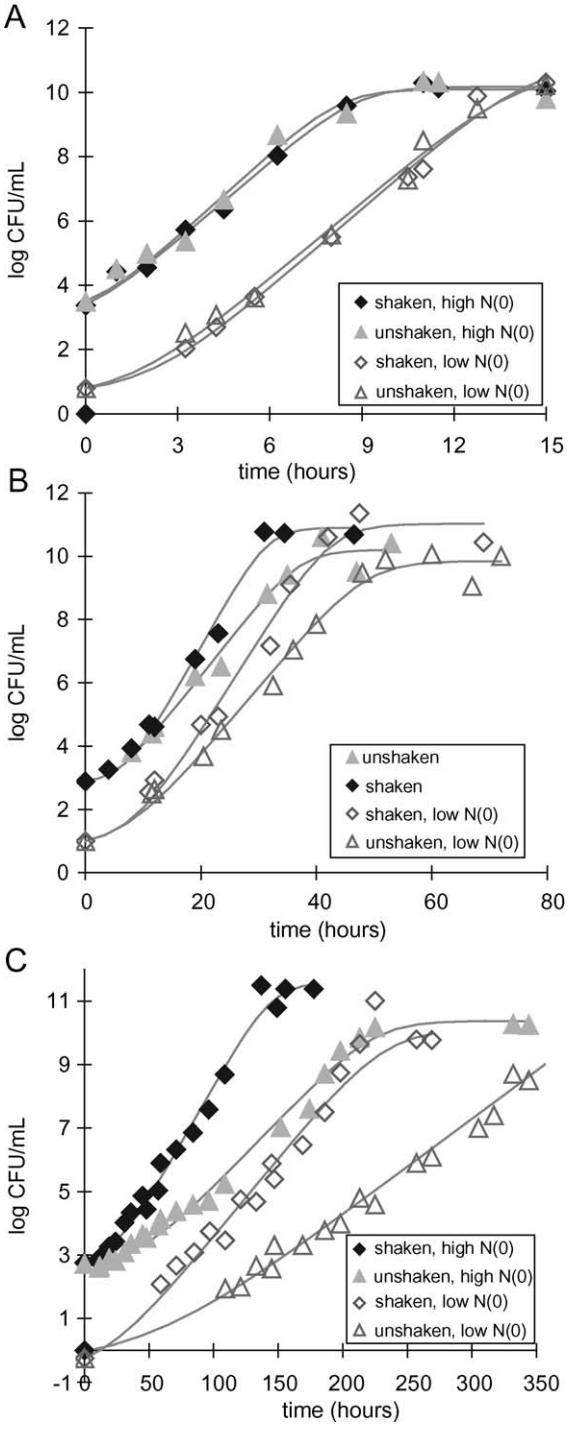

Fig. 1. Effects of temperature, agitation, and initial density of inoculum on growth of *E. coli* O157:H7 strain 1 in brain–heart infusion broth (pH 5.5). Lines represent fitted Baranyi models; symbols represent raw data from one of three replicate experiments. Incubation temperatures: (A) 37 °C; (B) 19 °C; (C) 10 °C. (♦) shaken, high initial density; (▲) unshaken, high initial density; (◇) shaken, low initial density; (△) unshaken, low initial density.

flexible sampling of the entire 24-h growth interval. The refrigerated storage of the working culture for up to 24 h did not alter the growth kinetics of this pathogen (data not shown), as has also been reported for related *Salmonella* spp. (Oscar, 1998). Both *E. coli* O157:H7 and *Salmonella* do not grow at 4 °C. In addition, experiments were conducted with and without agitation in the flask system using an intermediate density of strain 1 and two additional strains (2 and 3) to address potential strain variability at the incubation temperatures tested in the study (10, 19, and 37 °C).

A 2 × 2 design was selected to explore effects of lower pH at 10 °C in the flask system utilized by Buchanan et al. (1993). The initial densities (as described above) and pH (5.5 or 4.6) were factors further tested with strain 1.

For the factorial designs, 10 to 25 observations were obtained for each treatment over the full range of

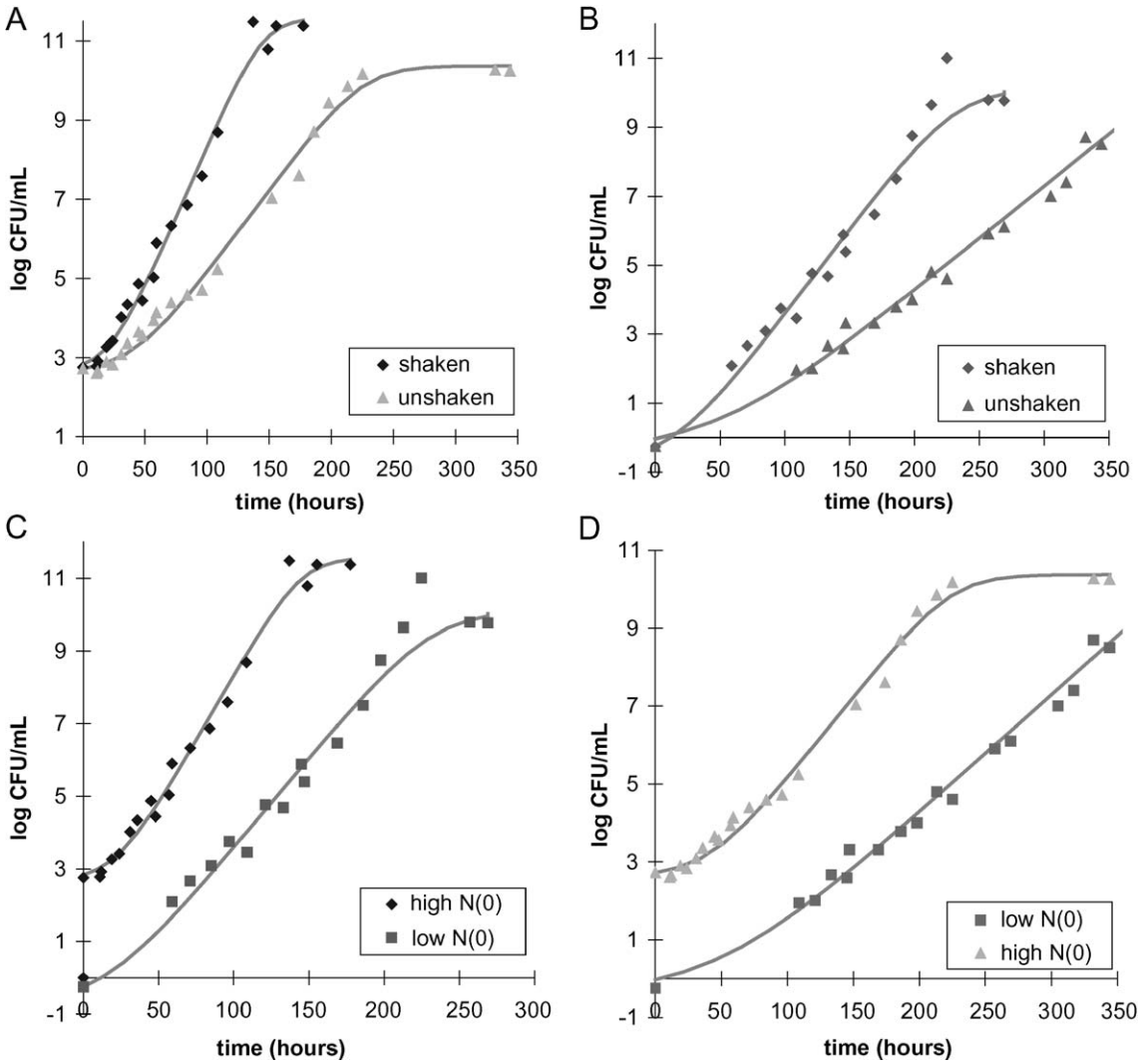

Fig. 2. Effects of agitation and initial density of inoculum on growth of *E. coli* O157:H7 strain 1 in brain–heart infusion broth (pH 5.5) at 10 °C. Lines represent fitted Baranyi models; symbols represent raw data from one of three replicate experiments. (A) High initial density, shaken vs. unshaken; (B) low initial density, shaken vs. unshaken; (C) shaken, high vs. low initial density; (D) unshaken, low vs. high initial density.

the growth curve at each incubation temperature. At least six observations per treatment were obtained for the multi-strain experiments. After each time interval at all temperatures, culture flasks were swirled (three times each in clockwise and counterclockwise directions) to ensure homogeneous sampling, and 50 or 100 μl sample aliquots from the culture flasks were plated undiluted or at appropriate dilutions in 0.1% peptone water onto four tryptic soy agar (TSA; Difco, Becton Dickinson Microbiology Systems) plates using a spiral plater (Autoplate 4000, Advanced Instruments, Norwood, MA). Plates were incubated for 15 h at 37 °C or for 36 h at room temperature. Numbers of colonies did not differ at these incubation temperatures. Direct plate counts ($\log_{10}$ CFU/ml) were estimated and recorded using an automated colony counter (Q-Count Advanced Instruments). Relative standard errors of $\log_{10}$ CFU/ml counts were generally below 5% for spiral plates.

Two designs were developed for qualitative screening assays of the growth/no growth interface for the nine strains using a microtiter plate format. The first study design included inoculation of triplicate microtiter plates with 20 μl of dilutions of each individual strain into 200-μl BHI at pH levels of 4.6–5.5. Initial densities of approximately 1–100 per well were achieved. Plates were incubated unshaken at 10, 19, or 37 °C for up to 2 weeks. Growth/no growth was visually assessed in duplicate experiments. Growth in the unshaken format was not evident as turbidity, rather as pellets of cells. The second study design included inoculation of 20 μl of dilutions of strains 1, 2, 8, and 9, at two or three different dilutions into 200-μl BHI at pH 5.5 and 5.0. Initial densities achieved in these experiments were approximately 50–100 per well to ensure observation of pellets within 2 weeks. These strains were selected for further study because growth kinetics differed. Plates were incubated at 10 °C for 2 weeks for visual assessment of growth/no growth. In addition, three wells per plate were selected for bacterial enumeration as spread plates. Cells in selected wells were suspended by gentle pipetting. Aliquots (10 μl) of the suspensions (undiluted or diluted) were applied to four quadrants of TSA plates, distributed in a cross pattern over the quadrant by gently tipping or rotating the plate, and the colonies enumerated. Growth of the four strains after 2 weeks of incubation at 10 °C was assessed by qualitative visual observation and quantitative methods described.

Table 1
Least squares means for linear regression slope and Baranyi lag, rate, and MPD parameters for interactions of temperature, agitation, and initial density for *E. coli* O157:H7 strain 1

Treatments						
Temperature (°C)	Agitation	Initial density	Mean slope[a]	Mean lag[b]	Mean rate[b]	Mean MPD[b]
10	unshaken	low	0.028^{A}	60^{A}	0.032^{A}	10.0^{A}
10	unshaken	high	0.037^{B}	40^{B}	0.043^{A}	9.6^{A}
10	shaken	low	0.049^{C}	27^{C}	0.056^{A}	10.1^{A}
10	shaken	high	0.059^{D}	18^{D}	0.065^{A}	11.6^{B}
19	unshaken	low	0.189^{A}	7.4^{A}	0.222^{A}	9.6^{A}
19	unshaken	high	0.204^{A}	5.7^{A}	0.230^{A}	10.0^{A}
19	shaken	low	0.246^{B}	6.7^{A}	0.275^{A}	10.9^{B}
19	shaken	high	0.259^{B}	5.8^{A}	0.306^{A}	11.0^{B}
37	unshaken	low	0.799^{A}	1.3^{A}	0.852^{A}	10.3^{A}
37	unshaken	high	0.758^{A}	1.2^{A}	1.040^{A}	10.7^{A}
37	shaken	low	0.801^{A}	0.4^{A}	0.852^{A}	9.9^{B}
37	shaken	high	0.793^{A}	0.4^{A}	0.898^{A}	10.0^{B}

Parameter means with different superscript letters within a temperature grouping were significantly different by Student's *t*-test ($\alpha = 0.05$) with two degrees of freedom.

[a] Linear regression.

[b] Baranyi model.

2.4. Curve-fitting and statistical analysis

For each treatment, kinetic data from experiments conducted in culture flasks were fit to simple linear regression (Excel, version 7, Microsoft, Seattle, WA) and the Baranyi (DMFit; Baranyi and Roberts, 1994) models. Two model forms were selected to address model and parameter uncertainty and parsimony of importance to risk assessors. Two parameters, slope and intercept, were estimated by simple linear regression for the exponential phase of the growth curves, excluding observations in the lag and stationary phases of the curves. For the six-parameter Baranyi model, three parameters were fixed (estimated initial density [y_0] and two curvature parameters, $n_{curve}=1$ and $m_{curve}=10$) in the DMFit routine, and three

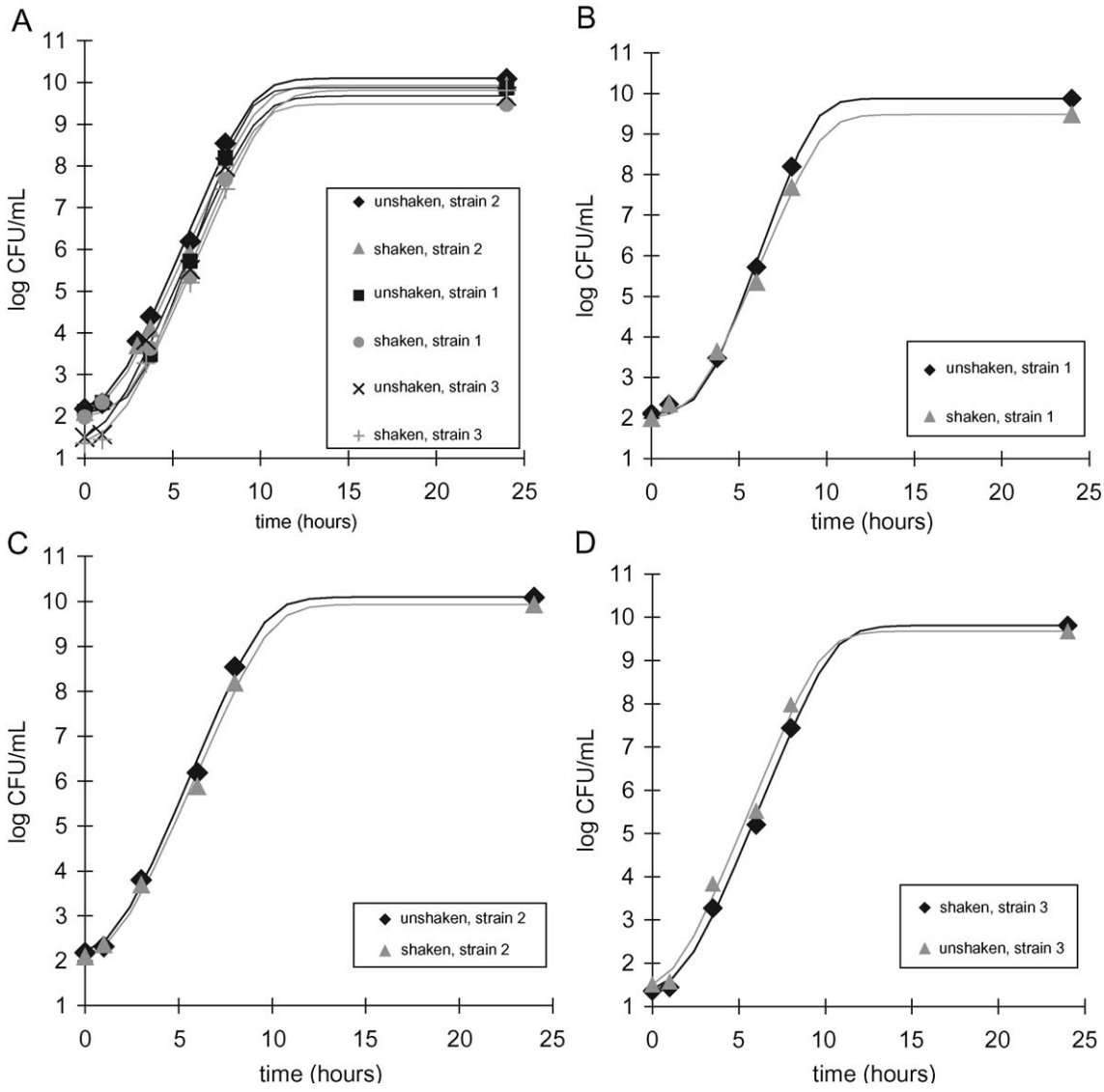

Fig. 3. Strain variability of growth kinetics at 37 °C under two conditions of agitation. (A) Strains 1, 2, and 3. (B) Strain 1. (C) Strain 2. (D) Strain 3.

parameters (lag, rate, y_{end} or MPD) were estimated from the system of differential equations described by Baranyi and Roberts (1994).

Analysis of variance (ANOVA) procedures (SAS, version 8, Cary, NC) were applied to estimated linear model parameters slope and intercept, and Baranyi model parameters rate, lag, and MPD for triplicate independent experiments conducted at the three incubation temperatures. Least squares means were compared using the Student's *t*-test for significant effects identified in the ANOVA.

3. Results

Both linear regression and Baranyi models provided good statistical fits to the data. Representative plots of fitted Baranyi models and the raw data for four con-

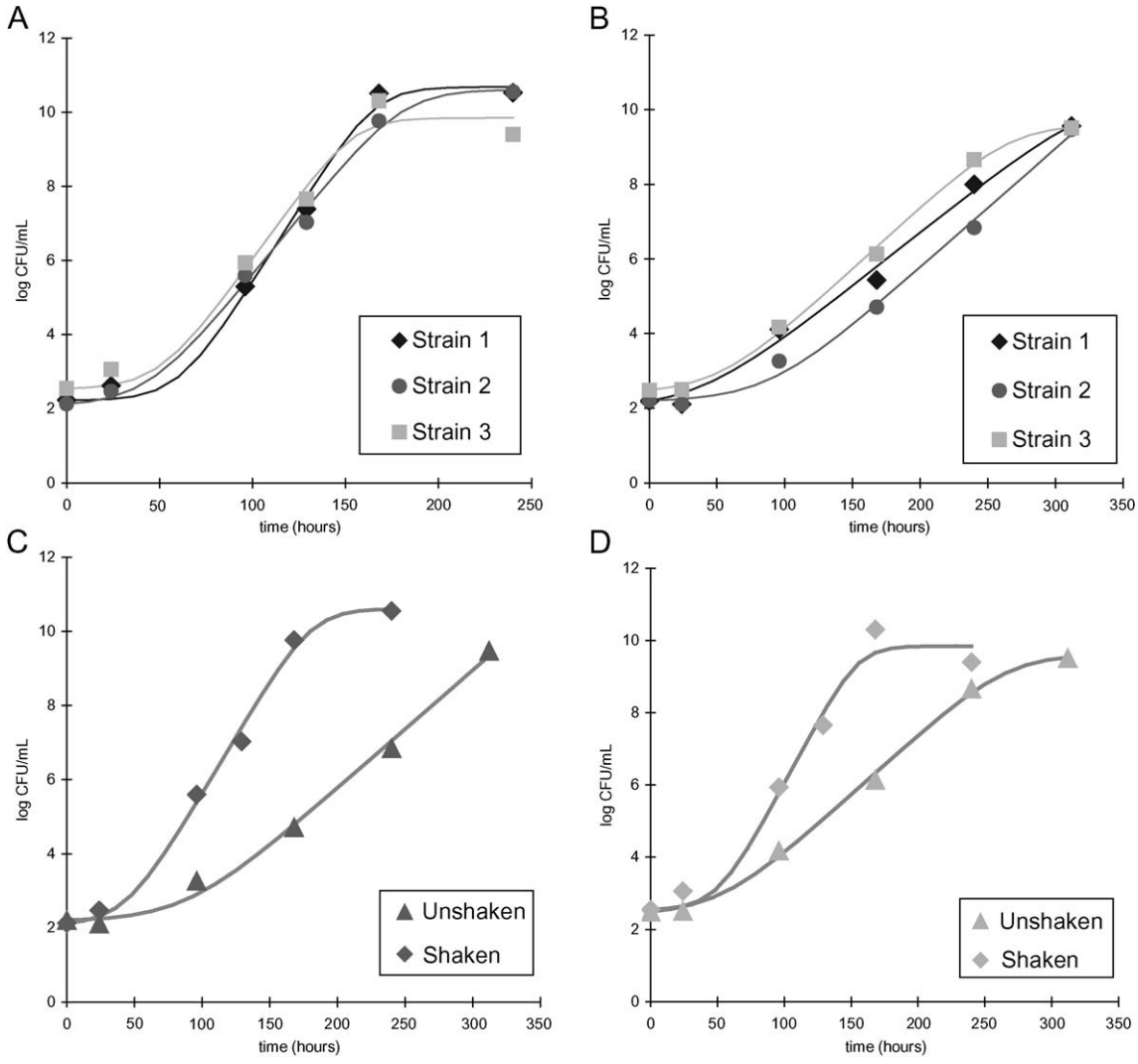

Fig. 4. Strain variability of growth kinetics at 10 °C under two conditions of agitation. (A) Strains 1, 2, and 3 shaken. (B) Strains 1, 2, and 3 unshaken. (C) Strain 2 shaken and unshaken. (D) Strain 3 shaken and unshaken.

ditions of agitation and initial density of inoculum are presented in Figs. 1 and 2. The patterns and magnitude of treatment effects were consistent across experiments. At a qualitative level, differences among treatments were negligible at 37 °C (Fig. 1A), obvious at 10 °C (Fig. 1C), and intermediate at 19 °C (Fig. 1B).

The significant effects by analysis of variance for Type I and Type III errors associated with the slope parameter of the linear portions of microbial growth curves included: (1) experiment; (2) temperature; and (3) agitation (probability of greater F statistic ≤ 0.02). Growth kinetics differed significantly by temperature for the Baranyi rate and lag parameters ($Pr>F<0.0001$). In addition to significant effects for temperature, the lag parameter was associated with significant effects for agitation, agitation/temperature interaction, initial density, initial density/temperature interaction, and experimental replication ($Pr>F<0.0001$). Temperature did not impose a statistically significant effect on the Baranyi MPD parameter. Significant effects associated with MPD were agitation, temperature/agitation interaction, initial density, and interaction of agitation, initial density, and temperature ($Pr>F<0.031$).

Results from statistical testing for four model parameters, summarized in Table 1, supported significant differences for slope and lag parameters due to all four treatments of agitation and initial density at 10 °C, for slope and MPD due to agitation treatments at 19 °C, and for MPD due to agitation at 37 °C. The trends of slope and Baranyi rate parameters were consistent at 10 and 19 °C. However, only the more extreme differences, such as the comparison of unshaken, low initial density and shaken, high initial density treatments, were statistically significant for the Baranyi rate parameter. When the kinetic patterns of one strain were well characterized, two additional strains tested demonstrated consistent patterns of growth under the two conditions of agitation. As noted previously at 37 °C, differences between agitation treatments were negligible for the additional strains (Fig. 3). However, a consistent pattern of growth by treatment at 10 °C was observed for all three strains tested, with marked alteration of the shape and position of the growth curves as the minimum growth temperature was approached (Fig. 4).

The curves included well-defined regions of linearity when lag and stationary phase observations were excluded, particularly for the 10 °C experiments including 13–25 observations within the linear region. The slopes derived using linear regression were consistently lower than the Baranyi rate parameters across treatments in replicate experiments. For each experimental replicate at 10 °C, the slopes differed significantly for all four treatments (Table 1). The slopes representing the shaken high initial density condition typical of most predictive microbiology experiments were 2-fold higher than the slopes for the unshaken low initial density treatments more typical of foods. Although the same trend was observed for the Baranyi

Table 2
Fitted parameters for linear regression and Baranyi models of *E. coli* O157:H7 strain 1 growth at 10 °C for four conditions of agitation and initial density

Shaken	Inoculum class	Estimated inoculum[a]	Experiment	Linear regression			Baranyi model			
				Slope	y intercept	r^2	Rate	Lag	MPD	r^2
No	low	−0.3	1	0.028	−1.317	0.98	0.033	62	11.4	0.97
No	low	0.2	2	0.025	−0.307	0.98	0.029	54	10.1	0.97
No	low	1.0	3	0.030	−0.540	0.95	0.034	64	8.6	0.97
No	high	2.9	1	0.036	1.790	0.98	0.042	43	10.4	0.99
No	high	3.5	2	0.039	2.433	0.97	0.043	42	10.2	0.99
No	high	3.0	3	0.037	2.203	0.94	0.044	35	8.3	0.97
Yes	low	−0.3	1	0.050	−1.299	0.96	0.052	27	10.4	0.96
Yes	low	0.2	2	0.046	−0.305	0.98	0.048	16	10.8	0.98
Yes	low	1.0	3	0.052	−0.077	0.92	0.069	37	9.2	0.93
Yes	high	2.8	1	0.064	1.9	0.98	0.070	21	11.6	0.99
Yes	high	3.7	2	0.056	3.169	0.99	0.059	13	11.4	0.99
Yes	high	3.0	3	0.058	2.422	0.93	0.065	19	11.7	0.95

[a] Units are: $\log_{10}$ CFU/ml estimated inoculum, slope, rate, and MPD; and hours for lag.

rate parameter at 10 °C (Table 2), the differences between treatments were not significantly different for all four treatments (Table 1).

The growth/no growth interface observed in microtiter plate assays was temperature dependent. All nine strains inoculated at initial densities of approximately 1.7 $\log_{10}$ CFU/well (50 CFU/well) formed visible cell pellets at pH 4.6–5.5 at 37 and 19 °C in replicate experiments after 7 days of static incubation. By 14 days at 10 °C, all nine strains formed visible pellets at pH 5.5, 5.4, and 5.2, and none of the nine strains grew at or below pH 4.8. However, qualitative differences at pH 5.0 were observed among strains in initial experiments within 14 days at 10 °C. Visible growth at pH 5.0 for strains 2 and 8 was not observed, while visible growth was observed for the remaining seven strains.

Quantitative measurement of growth and survival of strain 1 as a function of pH was verified in the flask system under unshaken conditions at 10 °C. The pathogen at high initial inoculum (3.1 $\log_{10}$ CFU/ml) at pH 4.6 declined 0.25 $\log_{10}$ CFU/ml within the first day (Baranyi rate = − 0.002), and survived without further decline for the remaining sampling intervals up to 12 days postinoculation. The pathogen at low initial inoculum (1 $\log_{10}$ CFU/ml) grew only at pH 5.5 (data not shown), and was not detected at pH 4.6 throughout the 12-day sampling period for this experiment.

Qualitatively, visible growth at pH 5.0 in subsequent microtiter plate assays was inconsistently observed within 14 days at 10 °C for strains 2 and 8 (Table 3). Quantitatively, strain 2 was the slowest growing strain among the four strains tested. Strain 2 increased 1.1 $\log_{10}$ units in the first experiment and increased 2.5 $\log_{10}$ units in a replicate experiment from nearly identical initial densities. In contrast, strain 1, the fastest growing strain, increased 6.5 and 7.2 $\log_{10}$ units by 14 days in replicate experiments. Strains 8 and 9 were more variable in growth for duplicate experiments, increasing after 14 days by 2 and 3.3 $\log_{10}$ units and 3.6 and 6.7 $\log_{10}$ units, respectively.

4. Discussion

The differences observed at 10 °C incubation in the shape and position of *E. coli* O157:H7 growth curves from the flask system for the four treatments of agitation and initial density in BHI at pH 5.5 (Fig. 2) are associated with all three growth parameters and interactions (lag, rate, and MPD). The significant effect of agitation and initial density at 10 °C was also observed for two additional strains tested (Fig. 3). However, at pH 5.5 typical of ground beef, even near the lower limit of growth temperature for *E. coli* O157:H7, strain variability for this panel of strains isolated from beef appeared as a relatively minor effect, compared to the significance of agitation and initial density treatments on kinetic parameters.

A number of factors may contribute to shorter lag and higher growth rate and MPD parameters in vigorously agitated liquid media. The cells under shaken conditions are less likely to remain clustered in microcolonies (Tsiamyrtzis et al., 2001) than expected under more typical unshaken conditions in food matrices. The major physiological impact of agitation may be due to greater availability of dissolved oxygen. Agitation of *E. coli* O157:H7 cocktails in BHI at pH 5.5 but under anaerobic atmosphere (Buchanan et al., 1993) resulted in growth rates

Table 3
Qualitative and quantitative observations for four *E. coli* O157:H7 strains in BHI (pH 5.0) after incubation at 10 °C for 14 days under unshaken conditions in replicate experiments

Strain	Inoculum level ($\log_{10}$ CFU/ml)[a]		Level at 14 days ($\log_{10}$ CFU/ml)		Increased growth over 14 days ($\log_{10}$ CFU/ml)	
	Replicate 1	Replicate 2	Replicate 1	Replicate 2	Replicate 1	Replicate 2
1	2.6	2.6	9.1 (++)[b]	9.8 (++)	6.5	7.2
2	2.6	2.6	3.7 (−)	5.1 (+/−)	1.1	2.5
8	2.5	2.7	5.8 (+)	4.7 (+/−)	3.3	2.0
9	2.4	2.7	6.0 (+)	9.4 (++)	3.6	6.7

[a] Average densities ($\log_{10}$ CFU/ml) were quantified from three wells per strain (coefficient of variations < 10%).
[b] Pellet size based on a scale of "+" to "++"; "−"= no pellet observed.

consistent with the unshaken high initial density treatment in this study.

The two model forms describe different aspects of growth rate. The slope represents the average rate of increase over extended periods of time, whereas the Baranyi rate parameter (Baranyi and Roberts, 1994) represents a potential maximal growth rate over a short time interval when growth rate is changing rapidly near the inflection points. The current study design ($n=3$ experiments) was sufficient to demonstrate statistical significance of the slope parameters for all four treatments at 10 °C (Table 1). However, the Baranyi growth rate parameter was not significantly different for all four treatments. The Baranyi growth rate parameter varied between experiments to a greater extent than the linear regression slope. The parametric test statistic selected had only two degrees of freedom for judging the significance of effects among the triplicate experiments. Higher numbers of replicate experiments at 10 °C may be necessary to demonstrate with more certainty the statistical significance of the Baranyi growth rate.

Higher initial densities of bacteria were theoretically associated with higher likelihoods of including at least one cell in the proper physiological state for immediate growth, without any lag time for adjustment (Baranyi, 1998). Therefore, the observation of longer lags at low initial density was not a surprising result. However, an unexpected observation of this study is evidence for density-dependence of growth rates at suboptimal conditions, such as low temperature and acid pH. Kaprelyants and Kell (1996) suggested that growth kinetics of bacterial populations may be dependent on initial density and communication between cells, as has been demonstrated for eukaryotic cells. A related theory of "quorum sensing" (Surette et al., 1999; Parsek and Greenberg, 2000) links regulation of gene expression to changes in bacterial cell density, and subsequently to coordinated behavior based on threshold densities of the population of bacterial cells including *E. coli* O157:H7. Gast and Holt (2000) observed density-dependence of growth for the related enteropathogen *Salmonella enteritidis* under certain conditions in experimentally inoculated eggs. In contrast, no data are available from studies inoculating nonsterile ground meat products with low densities of either of these pathogens. The ground beef matrix poses more significant methodological limitations for direct quantitation of pathogens in order to assess another major factor influencing the growth of pathogens in these food matrices: competition of small numbers of pathogen cells with the numerically and ecologically dominant indigenous microbiota adapted to refrigeration temperatures (Table 4). Risk assessors cannot account for the uncertainty of extrapolation from broth kinetic models without bridging studies or fully validated models generated in food matrices.

The 10 °C incubation temperature represents the 99th percentile of home refrigeration temperatures from a large national survey (Audits International, 1999). The multi-factorial nature of bacterial growth as evidenced by many interactions of correlated factors significant in this and other studies suggests that effects are unlikely to be consistent with simple linear adjustments of broth culture models. If no adjustment is made for the influence of agitation at high initial density in this study, growth is likely to be overpredicted at 10 °C by 4.9–5.9 $\log_{10}$ CFU/ml (76,000–769,000 CFU/ml) after 50 h and 8.1–8.8 $\log_{10}$ CFU/ml (1.3×10^8–6.3×10^8 CFU/ml) by 100 h. If no adjustment is made for the influence of agitation at low initial density, growth is unlikely to be detected after 50 h at 10 °C, based on results for treatments at low initial density under static conditions for two of three experiments. After 100 h at 10 °C without adjustment for agitation, absolute growth

Table 4
Comparison of maximum specific growth rate in culture broth at pH 5.5 for *Pseudomonas* spp. as the dominant organism (derived from Pin and Baranyi, 1998) and a cocktail of *E. coli* O157:H7 strains (95% confidence interval, PMP version 5.1)

Incubation temperature (°C)	*Pseudomonas*	*E. coli* O157:H7
2	0.08[a]	Not Tested
5	0.12	Below growth minimum[b]
10	0.24	0.038, 0.060

[a] $\log_{10}$ CFU/h.

[b] The minimum temperature for growth of *E. coli* O157:H7 at pH 5.5 was 10 °C (Buchanan et al., 1993). Buchanan reported no growth at pH 5.5 for incubation temperatures of 5 and 8 °C, defined as less than or equal to 1 $\log_{10}$ CFU/ml increase by 250 h of incubation.

for low initial density treatments is overpredicted by 2.2–3.1 $\log_{10}$ CFU/ml (9200–302,000 CFU/ml). The magnitude of overprediction is consistent with 16,000–135,000% overestimation of static growth potential for low initial densities. Ross et al. (2000) reported the extent of overestimations for extrapolation from broth culture models was 300–400% for growth rates in validation studies conducted in other foods. For low pH liquid commodities such as apple juice, overprediction bias for growth under static conditions and low temperature may also be significant. The impact of agitation for solid commodities is less relevant, but other food matrix factors may contribute to both variability and uncertainty that cannot be estimated from data or models on culture broth kinetics. The current study demonstrates the need for bridging studies or full validation studies in food matrices to determine the adjustments that are essential to characterize growth kinetics in food commodities, particularly for calculation of variability and uncertainty in exposure assessment models.

The FSIS currently recommends that consumers refrigerate raw ground beef for only 1 or 2 days prior to cooking (USDA, 2001) primarily on the basis of food quality. The need to communicate such a short refrigeration period for ground beef is based on scientific evidence from an extensive quantitative microbiological study conducted in fresh ground beef (USDA, 1996). All samples analyzed ($n=563$) were positive for Aerobic Plate Count (APC), which detects the spoilage microbiota. The 95% confidence interval for the mean APC reported in this study is 3.7–4.1 $\log_{10}$ CFU/g for fresh ground beef sampled at the grinder. In another study, APC counts in ground beef at retail markets were 5.0–6.5 $\log_{10}$ CFU/g (Ajjarapu and Shelef, 1999) before storage in consumer's refrigerators. Great uncertainty is associated with the initial density for the pathogen *E. coli* O157:H7 in positive samples of ground beef. The pathogen was not detected among 563 samples analyzed in the FSIS survey (USDA, 1996), and was detected at densities <15/g by most probable number methods in six samples of suspect ground beef from an outbreak (Marks, 1998).

In addition to numerical dominance in ground beef, the dominant spoilage organisms in refrigerated ground beef under aerobic conditions are pseudomonads (Pin and Baranyi, 1998), psychrotrophic organisms (Gill, 2002) adapted to growth at temperatures <15 °C. Gill (2002) also reported the effectiveness of commercial distribution and storage in preventing growth of mesophiles, such as *E. coli*, in properly chilled beef trimmings over an 18-day period. In addition to initiation of growth at lower temperatures, the pseudomonads continue to grow up to approximately 15 °C at faster rates than mesophiles such as *E. coli* O157:H7, once the 10 °C minimum growth temperature at pH 5.5 (Buchanan and Klawitter, 1992) is reached (Table 4).

Risk assessors have not fully incorporated the extensive body of evidence on microbial ecology of foods (ICMSF, 1980; Lebert et al., 2000; Walls, 1996; Brashears and Durre, 1999; Bredholt et al., 1999; Duncan et al., 1999; Ross, 1999, 2000; Kang and Fung, 1999, 2000; Takahashi et al., 1999; Vold et al., 2000; Nissen et al., 2000; Tamplin, in press) into exposure assessment models for food matrices such as meats (Cassin et al., 1998; Marks, 1998; Powell et al., 2000). Ross et al. (2000) described an ecological phenomenon termed the "Jameson Effect" as inhibition of growth of slow-growing organisms by the total density of all of the microbial populations present in foods. In addition, four studies provide some direct evidence that ecological factors of nonsterile ground beef will limit *E. coli* O157:H7 growth kinetics observed in more optimal conditions of broth-based pure culture experiments. For 10 °C, the Gompertz parameters for growth of *E. coli* O157:H7 in broth culture experiments reported in the Pathogen Modeling Program (Anonymous, 1996) include a 49-h lag, exponential growth rate of 0.05 log CFU/h, and maximal population density 9.3 log CFU/ml. In contrast, Walls (1996) estimated relative differences in Gompertz parameters for growth of this pathogen in nonsterile ground beef, including lag nearly half the time predicted, exponential growth rate 0.5-fold lower, and a 1–2 log CFU/g lower MPD at 12 °C. In addition, as temperature was further decreased to 10 °C, Tamplin (in press) observed immediate growth in nonsterile ground beef, without the 49-h lag predicted from broth models or the 12-h lag predicted at 12 °C by Walls et al. (1996). Further results at 10 °C in nonsterile ground beef include more than a 2-fold decrease in the Baranyi rate parameter and a 4-log CFU/g lower MPD than predicted in broth models (Tamplin, in press). Ross (1999) reported inhibition of MPD of *E. coli* in nonsterile minced beef. Vold et al. (1999) demonstrated

total inhibition growth of *E. coli* O157:H7 in three of four experiments in ground beef at 12 °C when the indigenous lactic acid bacteria were supplemented with a mixture of strains predominated by *Lactobacillus sakei*.

Similarly, Ushijima and Seto (1991) demonstrated a 4-$\log_{10}$/ml inhibition of the MPD of the enteropathogen *Salmonella typhimurium* in competition with five representative species of indigenous competitors in a continuous culture broth system. However, removal of any one of the five species of the indigenous competitors from the culture reduced the level of antagonism in the system. These results caution against simplifications of the complex community of competing bacterial species in foods without systematic study. Otherwise, Wiegert (1993) warns of the "condensation" problem, inappropriate selection of one or a few of the component species of the indigenous microflora that do not adequately represent the dynamics of the diverse natural communities interacting in the ecosystem. More targeted research will be needed to calibrate adjustments of available predictive microbiology models for risk assessment modeling of actual growth potential of low initial density mesophilic pathogens such as *E. coli* O157:H7, with the dense and diverse populations of psychotropic indigenous microbiota of nonsterile ground beef that are adapted to faster growth rates under refrigeration conditions.

Broth models might provide "fail-safe" predictions for long intervals of storage due to the influence of higher growth rate and MPD, but "fail-dangerous" predictions when researchers now document zero lag periods in ground beef matrices (Nissen et al., 2000; Vold et al., 2000; Tamplin, in press). Of particular importance for risk assessors will be the uncertainty in predictions of growth kinetics at the 1- to 2-day period recommended by FSIS for refrigerated storage in consumers' homes. If the estimates from Audits International (1999) for refrigerator temperatures are accurate (99% ≤ 10 °C), then growth of this pathogen is unlikely to occur with proper food handling procedures. One task of risk assessors and researchers will be the development of testable scenarios that depict potential deviations from proper handling to determine the probability and extent of growth in nonsterile ground beef that was temperature-abused.

Three publications depicting risk assessment of this pathogen in ground beef (Cassin et al., 1998; Marks, 1998; Powell et al., 2000), each considered direct extrapolation of the parameters generated from predictive microbiology experiments in pure culture to the more complex environment of nonsterile ground beef without adjustment for effects associated with the food matrix. These exposure assessment models permit maximum exponential growth of the pathogen to the maximal carrying capacities of up to 10^{11} CFU/g, representing densities per ml achieved under the optimal conditions in agitated pure culture broth. None of the outputs of these exposure assessments have been validated by independent scientific studies. In addition, these models do not take into account the full spectrum of errors described by Ross (1999), including homogeneity, completeness, model function, measurement, and numerical procedure errors. Thus, the plausibility of modeling exponential growth at rates observed in broth to densities greater than 10^{4-6} CFU/g appear questionable, even for high initial densities of pathogens (Ross, 1999; Tamplin, in press). Growth models for another foodborne pathogen, *Listeria monocytogenes*, in broth appeared to overpredict growth rates in food matrices by 300–400% (Ross et al., 2000), smaller levels of overprediction than estimated in this study for *E. coli* O157:H7.

In conclusion, the effects of agitation, initial density, pH, and strain were significant for growth kinetics near the boundaries of the growth/no growth interface for *E. coli* O157:H7. As Nauta (2002) suggests, additional research is required if risk assessors are to incorporate calculation of variability and uncertainty of pathogen growth into exposure assessment models. Microbial risk assessment processes are evolving to incorporate more science to replace judgements that do not hold up to hypothesis testing in controlled scientific experiments. The analytical-deliberative process described for risk analysis by the National Research Council (1996) is consistent with systematic analysis of the available bodies of evidence, such as kinetic data for broth cultures and nonsterile food matrices, to support microbial exposure assessment modeling. Although some kinetic data are available, additional research is needed to test conditions limiting pathogen growth in nonsterile foods. Validation of predictive microbiology models

generated in food matrices should be considered in development of strategic research initiatives targeting data gaps for microbial foodborne hazards. Adjustments appear to be needed to account for suboptimal growth kinetics in nonsterile foods.

Acknowledgements

The work was conducted at the University of Maryland Eastern Shore and included collaboration between the USDA Food Safety and Inspection Service and the USDA Agricultural Research Service, Eastern Regional Research Center. Drs. Kisun Yoon and Tom Oscar graciously provided laboratory space and access to equipment. Bacterial strains were generously provided by F. Beacon (FSIS, Athens, GA). Helpful insights on early versions of the manuscript were offered by Dr. Isabel Walls of the ILSI Risk Science Institute and members of the Biosciences Division of the USDA Food Safety and Inspection Service. The authors also acknowledge many helpful suggestions from two anonymous journal reviewers.

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